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<b>(21) International Application Number:</b> PCT/US99/24326 <b>(22) International Filing Date:</b> 19 October 1999 (19.10.99)  <b>(30) Priority Data:</b> 60/104,809              19 October 1998 (19.10.98)      US 09/282,239              31 March 1999 (31.03.99)              US  <b>(71) Applicant:</b> CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).  <b>(72) Inventors:</b> GOLDMAN, Steven, A.; 7 Hillcrest Court, South Salem, NY 10590 (US). WANG, Su; Apartment 31, 441 Lawn Avenue, Palisades Park, NJ 07650 (US).  <b>(74) Agents:</b> GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		<b>(81) Designated States:</b> CA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS  <b>(57) Abstract</b>  <p>The present invention is directed to a method of separating oligodendrocyte cells or progenitor cells thereof from a mixed population of cells. It comprises selecting a promoter which functions only in the oligodendrocyte cells or progenitor cells thereof, introducing a nucleic acid molecule encoding a fluorescent protein under control of that promoter into the mixed population cells, allowing the oligodendrocyte cells or progenitor cells thereof to express the fluorescent protein and separating the fluorescent cells from the mixed population cells, where the separated cells are the oligodendrocyte cells or progenitor cells thereof. The invention also relates to the isolated and enriched human oligodendrocyte cells or progenitor cells thereof.</p>		

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## A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS

5 This application claims the benefit of U.S. Provisional Patent  
Application Serial No. 60/104,809 filed October 19, 1998.

### FIELD OF THE INVENTION

10 The present invention relates generally to a method of separating cells  
of interest, in particular oligodendrocyte cells, from brain tissue.

### BACKGROUND OF THE INVENTION

15 Throughout this application various publications are referenced, many  
in parenthesis. Full citations for these publications are provided at the end of the  
Detailed Description. The disclosures of these publications in their entireties are  
hereby incorporated by reference in this application.

20 The damaged brain is largely incapable of functionally significant  
structural self-repair. This is due in part to the apparent failure of the mature brain to  
generate new neurons (Korr, 1980; Sturrock, 1982). However, the absence of  
neuronal production in the adult vertebrate forebrain appears to reflect not a lack of  
appropriate neuronal precursors, but rather their tonic inhibition and/or lack of post-  
mitotic trophic and migratory support. Converging lines of evidence now support the  
contention that neuronal and glial precursor cells are distributed widely throughout  
25 the ventricular subependymal of the adult vertebrate forebrain, persisting across a  
wide range of species groups (Goldman and Nottebohm, 1983; Reynolds and Weiss,  
1992; Richards et al., 1992; Kirschenbaum et al., 1994; Kirschenbaum and Goldman,  
1995a; reviewed in Goldman, 1995; Goldman, 1997; Goldman, 1998; Goldman and  
Luskin, 1998; and Gage et al., 1995). Most studies have found that the principal  
30 source of these precursors is the ventricular zone (Goldman and Nottebohm, 1983;  
Goldman, 1990; Goldman et al., 1992; Lois and Alvarez-Buylla, 1993; Morshead et  
al., 1994; Kirschenbaum et al., 1994; Kirschenbaum and Goldman, 1995), though  
competent neural precursors have been obtained from parenchymal sites as well  
(Richards et al., 1992; Palmer et al., 1995; Pincus et al., 1998). In general, adult

progenitors respond to epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) with proliferative expansion (Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1995; Kuhn et al., 1997), may be multipotential (Vescovi et al., 1993; Goldman et al., 1996), and persist throughout life (Goldman et al., 1996). In rodents  
5 and humans, their neuronal daughter cells can be supported by brain-derived neurotrophic factor (BDNF) (Kirschenbaum and Goldman, 1995a), and become fully functional in vitro (Kirschenbaum et al., 1994, Pincus et al., 1998a, and Pincus et al. 1998b), like their avian counterparts (Goldman and Nedergaard, 1992).

A major impediment to both the analysis of the biology of adult neural  
10 precursors, and to their use in engraftment and transplantation studies, has been their relative scarcity in adult brain tissue, and their consequent low yield when harvested by enzymatic dissociation and purification techniques. As a result, attempts at either manipulating single adult-derived precursors or enriching them for therapeutic replacement have been difficult. The few reported successes at harvesting these cells  
15 from dissociates of adult brain, whether using avian (Goldman et al., 1992; 1996c), murine (Reynolds and Weiss, 1992), or human (Kirschenbaum et al., 1994) tissue, have all reported <1% cell survival. Thus, several groups have taken the approach of raising lines derived from single isolated precursors, continuously exposed to mitogens in serum-free suspension culture (Reynolds and Weiss, 1992; Morshead et  
20 al., 1994; Palmer et al., 1995). As a result, however, many of the basic studies of differentiation and growth control in the neural precursor population have been based upon small numbers of founder cells, passaged greatly over prolonged periods of time, under constant mitogenic stimulation. The phenotypic potential, transformation state and karyotype of these cells are all uncertain; after repetitive passage, it is  
25 unclear whether such precursor lines remain biologically representative of their parental precursors, or instead become transformants with perturbed growth and lineage control.

In order to devise a more efficient means of isolating native,  
unpassaged and untransformed progenitor cells from brain tissue, a strategy by which  
30 brain cells could be freely dissociated from brain tissue, then transduced in vitro with plasmid DNA bearing a fluorescent reporter gene under the control of neural progenitor cell-type specific promoters was developed (Wang et al., 1998). This

permitted isolation of the elusive neuronal progenitor cell of the CNS, using the T $\alpha$ 1 tubulin promoter, a regulatory sequence expressed only in neuronal progenitor cells and young neurons.

The repair of damaged brain requires not only sources of new neurons  
5 but also new glial support cells. Oligodendrocytes are the glial cell type that produce myelin and insulate neuronal axons by ensheathment with myelin-bearing cell processes. Like neurons, oligodendrocytes are largely postmitotic and cannot regenerate through proliferative expansion. However, persistent oligodendrocyte progenitors have been described in adult rodent subcortical white matter, and may  
10 provide a substrate for remyelination after demyelinating injury (Gensert, 1996; Gensert, 1997). In humans, the demonstration and identification of analogous subcortical oligodendrocyte progenitor cells has been problematic. A pre-oligodendrocytic phenotype has been described in adult human subcortical white matter, though these postmitotic cells may have included mature oligodendrocytes  
15 recapitulating their developmental program after dissociation (Armstrong, 1992). Rare examples of oligodendrocytes derived from mitotic division have also been reported in human subcortical dissociates (Scolding, 1995), but the identification and isolation of their mitotic progenitors have proven elusive. As a result, the enrichment of these cells for functional utilization has proven difficult. In particular, the cells  
20 have not been preparable in the numbers or purity required for *in vivo* engraftment into demyelinated recipient brain, whether experimentally or for clinical therapeutic purposes.

A strong need therefore exists for a new strategy for identifying, separating, isolating and purifying native oligodendrocyte precursor cells from brain  
25 tissue. Such isolated, enriched native precursors may be used in engraftment and transplantation in demyelinating disorders, as well as for studies of growth control and functional integration.

## SUMMARY OF INVENTION

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To this end, the subject invention provides a method of separating Oligodendrocytes or progenitor cells thereof from a mixed population of cells, based

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upon cell type-selective expression of cell-specific promoters. This method includes selecting a promoter which functions only in the oligodendrocyte cells or progenitor cells thereof, then introducing a nucleic acid molecule encoding a fluorescent protein under control of that promoter into the mixed population of cells, allowing the  
5 oligodendrocyte cells or progenitor cells thereof to express the fluorescent protein, and separating the fluorescent cells from the mixed population of cells, where the separated cells are the oligodendrocyte cells or progenitor cells thereof.

Another aspect of the invention relates to the isolated oligodendrocyte cells or progenitors thereof.

10 A promoter is chosen which specifically drives expression in oligodendrocyte cells or progenitor cells thereof but not in other cells of the nervous system. The fluorescent protein will therefore only be expressed and detectable in cells in which the promoter operates, i.e. those cells for which the promoter is specific.

15 The method involves the introduction of nucleic acid encoding the fluorescent protein, under the control of the cell specific promoter, into a plurality of cells. Various methods of introduction known to those of ordinary skill in the art can be utilized, including (but not limited to) viral mediated transformation (e.g., adenovirus mediated transformation), electroporation, and liposomal mediated  
20 transformation.

After cell specific expression of the fluorescent protein, such as green fluorescent protein (GFP), the cells expressing the fluorescent protein are separated by an appropriate means. In particular, the cells can be separated by fluorescence activated cell sorting. The method of the subject invention thus provides for the  
25 enrichment and separation of the oligodendrocyte cells or progenitor cells thereof.

Contemporary approaches toward the use of neural precursor cells have focused upon preparing clonal lines derived from single progenitors. However, such propagated lines can become progressively less representative of their parental precursors with time and passage in vitro. To circumvent these difficulties, the  
30 method of the subject invention provides a strategy for the live cell identification, isolation and enrichment of native oligodendrocyte precursors and their oligodendrocyte daughter cells, by fluorescence-activated cell sorting of subcortical

white matter cells transfected with fluorescent protein, driven by the oligodendrocyte-specific early promoter (P2) for cyclic nucleotide phosphodiesterase (CNP) (this promoter is hereinafter referred to as P/CNP2). Using this approach, oligodendrocyte precursors and their young oligodendrocyte daughters can be identified and  
5 selectively harvested from a wide variety of samples, including embryonic and adult brain of avian, mammalian, and human origin. This approach allows for the enrichment of neural precursors from both adults and embryos, with a yield substantially higher than that achievable through standard techniques of selective dissection and differential centrifugation. CNP protein, 2',3'-cyclic nucleotide 3'-  
10 phosphodiesterase (CNP; EC 3.1.4.37) (Tsukada, et al., 1992; Vogel et al., 1988, which are hereby incorporated by reference), is one of the earliest known myelin-associated proteins to be synthesized in developing oligodendrocytes. It is expressed by newly generated cells of oligodendrocytic lineage even within the ventricular zone, and appears to be expressed by their precursors as well, in both rodents and humans  
15 (Scherer et al., 1994, Yu et al., 1994; Peyron et al., 1997; Grever et al., 1997, which are hereby incorporated by reference). Importantly, the 5' regulatory region of the CNP gene includes 2 distinct promoters, P2 and P1, that are associated with 2 distinct RNAs, and are sequentially activated at different developmental stages (Douglas et al., 1992; Douglas and Thompson, 1993; Monoh et al., 1993; Scherer et al., 1994,  
20 which are hereby incorporated by reference) (Fig. 1). Only the CNP mRNA transcribed from the more upstream promoter, or P2 is found in the fetal brain, suggesting that the P2 promoter (P/CNP2) directs expression to young Oligodendrocytes and their precursors (Gravel et al., 1998; O'Neill et al., 1997, which are hereby incorporated by reference). As a result, the CNP2 promoter was chosen  
25 for this study for its ability to target transgene expression to oligodendrocyte progenitors and their immature progeny.

Extension of this approach to include fluorescent transgenes under the control of stage- and phenotype-specific promoters (both of which are intended to be covered by reference to "cell-specific" promoters herein) allows even more specific  
30 separations to be performed, for example, of oligodendrocytes over a range of developmental stages. More broadly, this strategy may be applied to any tissue of interest for which a cell-specific promoter is available, thereby allowing the

identification, isolation and separation of progenitor cells and their products from any tissue for which constituent cells have been assigned phenotype-selective promoters. This strategy permits sufficient enrichment for in vivo implantation of the defined and separated progenitor pools, as well as for in vitro analyses of phenotypic specification and growth control.

By providing a means of identifying oligodendrocytic precursors while alive, even when present in small numbers in mixed populations, the use of fluorescent transgenes driven by cell type-selective promoters such as P/CNP2 will allow the specification of phenotype to be studied and perturbed on the single cell level, an approach that had previously only been feasible on larger populations. Indeed, when used in conjunction with post-transfection FACS, this strategy may permit the enrichment of any cell type for which stage- or phenotype-specific promoters are available. For instance, similar GFP constructs based upon early neuronal promoters, such as T $\alpha$ 1 tubulin (Wang et al., 1998), might similarly permit the enrichment of neuronal as well as oligodendrocytic precursors from adult brain tissue. As a result, spectrally distinct GFP variants with non-overlapping emission spectra (Heim and Tsien, 1996), each driven by a different cell-specific promoter, will allow concurrent identification of neuronal and oligodendrocytic precursors in vitro. Multi-channel cell sorting based upon the concurrent use of several lasers with non-overlapping excitation lines, such as Ar-K and He-Ne, should then allow the separation and simultaneous isolation of several distinct precursor phenotypes from a given brain sample.

By providing a means to identify and enrich viable oligodendrocytic precursor cells, from adult as well as fetal brain, this strategy may allow a significant acceleration in the study of precursor and stem cell biology. In particular, it may allow the preparation and enrichment of both oligodendrocytic and other neural precursor cells in sufficient number to permit implantation and engraftment using native, unpassaged adult-derived progenitor cells. This approach may spur the development of induced adult oligoneogenesis as a viable therapeutic modality for the structural repair of the damaged or demyelinated central nervous system, whether in the brain or spinal cord. In particular, the potential use of these cells as substrates for induced remyelination, whether upon endogenous activation or engraftment, suggests

therapeutic strategies appropriate to a variety of white matter diseases. These potential therapeutic targets include ischemic demyelination, as in subcortical lacunar infarction and hypertensive leukoencephalopathy, post-inflammatory demyelinations, such as radiation necrosis and remitted multiple sclerosis, as well as the degenerative and metabolic leukodystrophies.

### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figure 1 shows the human P/CNP2:hGFP vector. Humanized GFP (Levy, et al., 1996), a mutant form of red-shifted GFP optimized for expression in human cells, was placed under the control of the human CNP2 promoter (P/hCNP2; Gravel, et al., 1996). To construct P/hCNP2:hGFP, an XbaI-XbaI fragment encompassing exon 0 and part of intron 1 was isolated from the SK/hgCNP plasmid, which contains the complete sequence of the human CNP gene (Gravel, et al., 1996), and was then linked to the hGFP gene fused to the SV40 polyadenylation signal.

Figures 2A-I show that adult human white matter harbors oligodendrocyte progenitors. Immunocytochemistry of white matter dissociates for a panel of cell type-selective antigens revealed a diverse representation of phenotypes before sorting. Figures 2A-C show a typical bipolar cell, double labeled for A2B5 (red) and BrdU (yellow), fixed after 4 DIV. Figures 2D-F show a cluster of postmitotic O4<sup>+</sup> cells (Figures 2D-E) and an overtly less mature BrdU-incorporating O4<sup>+</sup>/BrdU<sup>+</sup> cell (Figure 2F), all fixed after 7 DIV. Figures 2G-I show representative examples of the diverse phenotypes present in the adult white matter. These included cells expressing CNP (Figure 2G), GFAP (Figure 2H), and TuJ1 (Figure 2I)-immunoreactivities, which respectively identify Oligodendrocytes, astrocytes, and neurons; each cell type was found in the proportion noted in the text.

Figure 3 shows that P/hCNP2:hGFP identifies a population of bipolar, A2B5<sup>+</sup> cells. GFP expression was observed within 4 to 5 days after transfection. The P/hCNP2:hGFP<sup>+</sup> cells typically first appeared as small, bipolar cells. Figures 3A-F show P/hCNP2:hGFP-expressing cells (Figures 3A, C, E) and their corresponding

phase contrast micrographs (Figures 3B, D, F). Figures 3G-H show immunocytochemistry identified the P/hCNP2:hGFP<sup>+</sup> bipolar cells as A2B5<sup>+</sup>. The inset in Figure 3H shows that this cell incorporated BrdU.

Figure 4 shows that culturing, isolation, and enrichment of oligodendrocyte progenitors. Adult human subcortical white matter, derived from surgical samples of frontal and temporal lobe, was dissected and enzymatically dissociated using papain and DNase, then cultured and transfected with either P/hCNP2:hGFP or control plasmids (hP/CMV:hGFP and P/hCNP2:*lacZ*).

Figures 5A-D show that isolation of P/hCNP2:hGFP<sup>+</sup> cells by FACS. Figures 5A-B show a representative sort of a human white matter sample, derived from the frontal lobe of a 42 year-old woman during repair of an intracranial aneurysm. This plot shows 50,000 cells (sorting events) with their GFP fluorescence intensity plotted against their forward scatter (a measure of cell size). Figure 5A indicates the result obtained from a nonfluorescent P/hCNP2:*lacZ*-transfected control, while Figure 5B indicates the corresponding result from a matched culture transfected with P/hCNP2:hGFP. Figures 5C and D show phase and fluorescence images of GFP<sup>+</sup> cells before (Figure 5C) and 2 hrs after (Figure 5D) sorting.

Figures 6A-I show P/hCNP2:hGFP-sorted cells divide and express oligodendrocytic markers. Figures 6A-C show a bipolar A2B5<sup>+</sup>/BrdU<sup>+</sup> cell, 48 hrs after FACS. Figures 6D-F show that within 3 weeks the bipolar cells matured into fibrous, O4<sup>+</sup> cells. These cells often incorporated BrdU, indicating their *in vitro* origin from replicating A2B5<sup>+</sup> cells. Figures 6G-I show a multipolar oligodendrocyte expressing CNP, still expressing GFP 3 weeks after FACS.

Figure 7A-F show FACS-sorted P/hCNP2:hGFP<sup>+</sup> cells mature largely as oligodendrocytes. Figures 7A-B show P/hCNP2:hGFP-sorted cells express O4 (*red*) and begin process elaboration within 4 days after FACS. Figures 7C-D show that by 2 weeks post-FACS, these cells generally develop multipolar morphologies. *red*., O4-immunoreactive cells. Figures 7E-F show that progenitor derived-cells matured further over the following weeks, developing oligodendrocytic morphologies and both CNP protein and galactocerebroside expression by 4 weeks *in vitro*.

**DETAILED DESCRIPTION**

A plasmid designated pGFP10.1 has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International  
5 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 75547 on September 1, 1993. This plasmid is commercially available from the ATCC due to the issuance of U.S. Patent No. 5,491,084 on February 13, 1996 in which the plasmid is described.  
10 This plasmid comprises a cDNA which encodes a green fluorescent protein (GFP) of *Aequorea victoria* as disclosed in U.S. Patent No. 5,491,084 to Chalfie et al., the contents of which are incorporated herein by reference.

The plasmid designated pT $\alpha$ 1-RSGFP has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International  
15 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 98298 on January 21, 1997. This plasmid uses the red shifted GFP (RS-GFP) of Clontech Laboratories, Inc. (Palo Alto, California), and the T $\alpha$ 1 promoter sequence provided by Dr. F. Miller (Montreal  
20 Neurological Institute, McGill University, Montreal, Canada). In accordance with the subject invention, the T $\alpha$ 1 promoter can be replaced with another specific promoter, and the RS-GFP gene can be replaced with another form of GFP, by using standard restriction enzymes and ligation procedures.

The plasmid designated pT $\alpha$ 1-hGFP has been deposited pursuant to,  
25 and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 98299 on January 21, 1997. This plasmid uses the humanized GFP (GFP<sub>h</sub>) of Zolotukhin and Muzyczka (Levy et al. 1996b), and the T $\alpha$ 1 promoter sequence provided by Dr. F. Miller (Montreal). In  
30 accordance with the subject invention, the T $\alpha$ 1 promoter can be replaced with another

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specific promoter, and the GFP<sub>h</sub> gene can be replaced with another form of GFP, by using standard restriction enzymes and ligation procedures.

The plasmid designated pCNP2-hGFP has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International  
5 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 98934 on October 20, 1998.

The plasmid designated pCNP(1 + 2)-hGFP has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the  
10 International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession No. 98935 on October 20, 1998.

As used herein, the term "isolated" when used in conjunction with a  
15 nucleic acid molecule refers to: 1) a nucleic acid molecule which has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), or 2) a nucleic acid molecule having the same nucleotide sequence but not necessarily separated from the organism (i.e. synthesized or recombinantly produced nucleic acid molecules).

20 The subject invention provides a method of separating oligodendrocyte cells or progenitor cells thereof from a mixed population of cells, based upon cell type-selective expression of cell specific promoters. This method includes selecting a promoter which functions only in the oligodendrocyte cells or progenitor cells thereof, introducing a nucleic acid molecule encoding a fluorescent protein under control of  
25 the promoter into the mixed population of cells, allowing the oligodendrocyte cells or progenitor cells thereof to express the fluorescent protein; and separating the fluorescent cells from the mixed population of cells, where the separated cells are the oligodendrocyte cells or progenitor cells thereof.

The cells of particular interest according to the subject invention are  
30 oligodendrocyte cells, more particularly oligodendrocyte precursor cells. Any cell which one desires to separate from a plurality of cells can be chosen according to the subject invention, as long as a promoter specific for the chosen cell is available.

“Specific”, as used herein to describe a promoter, means that the promoter functions only in the chosen cell type. A chosen cell type can refer to different types of cells, or different stages in the developmental cycle of a cell.

Illustrative of possible cell and promoter combinations which can be used in the subject invention are the following: a mature oligodendrocyte and a cyclic nucleotide phosphodiesterase I (i.e. CNPase I) promoter (Scherer et al. 1994); a myelinating oligodendrocyte and a myelin basic protein promoter (Wrabetz et al. 1993); an oligodendrocyte and a JC virus minimal core promoter (Krebs et al. 1995); a precursor and a JC virus minimal core promoter (Krebs et al., 1995); a myelinating oligodendrocyte and a proteolipid protein promoter (Cambi and Kamholz 1994); or an oligodendrocyte precursor and a cyclic nucleotide phosphodiesterase II (i.e. P/CNP2) promoter (Scherer et al. 1994). Preferably, the cell of interest is an oligodendrocyte or progenitor cell thereof and the promoter is a P/CNP2.

Having determined the cell of interest and selected a promoter specific for the cell of interest, a nucleic acid molecule encoding a fluorescent protein, preferably a green fluorescent protein, under the control of the promoter is introduced into a plurality of cells to be sorted.

The isolated nucleic acid molecule encoding a green fluorescent protein can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA, including messenger RNA or mRNA), genomic or recombinant, biologically isolated or synthetic. The DNA molecule can be a cDNA molecule, which is a DNA copy of a messenger RNA (mRNA) encoding the GFP. In one embodiment, the GFP can be from *Aequorea victoria* (U.S. Patent No. 5,491,084). A plasmid encoding the GFP of *Aequorea victoria* is available from the ATCC as Accession No. 75547. A mutated form of this GFP (a red-shifted mutant form) designated pRSGFP-C1 is commercially available from Clontech Laboratories, Inc. (Palo Alto, California).

Mutated forms of GFP that emit more strongly than the native protein, as well as forms of GFP amenable to stable translation in higher vertebrates, are now available and can be used for the same purpose. The plasmid designated pT $\alpha$ 1-GFP $\beta$  (ATCC Accession No. 98299) includes a humanized form of GFP. Indeed, any nucleic acid molecule encoding a fluorescent form of GFP can be used in accordance with the subject invention. Furthermore, any nucleic acid molecule encoding an

enzyme that can catalyze the conversion of a fluorogenic substrate to a fluorophore can be used in accordance with the subject invention. An example is the use of a cell-specific promoter to drive *lacZ* expression, with the detection and sorting of *lacZ*-expressing cells being by means of incubation with the fluorogenic substrates FDG (fluorescein- $\beta$ -D-galactopyranoside) or CMFDG (chloromethyl-FDG).

Standard techniques are then used to place the nucleic acid molecule encoding GFP under the control of the chosen cell specific promoter. Generally, this involves the use of restriction enzymes and ligation (see below).

The resulting construct, which comprises the nucleic acid molecule encoding the GFP under the control of the selected promoter (itself a nucleic acid molecule) (with other suitable regulatory elements if desired), is then introduced into a plurality of cells which are to be sorted. Techniques for introducing the nucleic acid molecules of the construct into the plurality of cells may involve the use of expression vectors which comprise the nucleic acid molecules. These expression vectors (such as plasmids and viruses) can then be used to introduce the nucleic acid molecules into the plurality of cells.

Various methods are known in the art for introducing nucleic acid molecules into host cells. These include: 1) microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles; 2) dextran incubation, in which DNA is incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell; 3) calcium phosphate coprecipitation, in which cells efficiently take in DNA in the form of a precipitate with calcium phosphate; 4) electroporation, in which cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures (passage through these vesicles may

sometimes destroy or damage DNA); 5) liposomal mediated transformation, in which DNA is incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm; 6) biolistic transformation, in which DNA is absorbed to the surface of gold particles and fired  
5 into cells under high pressure using a ballistic device; and 7) viral-mediated transformation, in which nucleic acid molecules are introduced into cells using viral vectors. Since viral growth depends on the ability to get the viral genome into cells, viruses have devised efficient methods for doing so. These viruses include retroviruses and lentivirus, adenovirus, herpesvirus, and adeno-associated virus.

10 As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular  
15 cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

In accordance with one of the above-described methods, the nucleic acid molecule encoding the GFP is thus introduced into a plurality of cells. The  
20 promoter which controls expression of the GFP, however, only functions in the cell type of interest. Therefore, the GFP is only expressed in the cell type of interest. Since GFP is a fluorescent protein, the cells of interest can therefore be identified from among the plurality of cells by the fluorescence of the GFP.

Any suitable means of detecting the fluorescent cells can be used. The  
25 cells may be identified using epifluorescence optics, and can be physically picked up and brought together by Laser Tweezers (Cell Robotics Inc., Albuquerque, New Mexico). They can be separated in bulk through fluorescence activated cell sorting, a method that effectively separates the fluorescent cells from the non-fluorescent cells (e.g., Wang et al., 1998).

30 The method of the subject invention thus provides for the isolation and enrichment of oligodendrocyte precursors from embryonic and adult brain of both fetal and adult, rodent and human derivation. Specifically, fluorescence-activated cell

sorting of subependymal and subcortical white matter cells transfected with green fluorescent protein driven by the oligodendrocyte P/CNP2 promoter is provided. CNPase is localized almost exclusively to the nervous system, within which it appears specific for oligodendrocytes (Scherer et al., 1994). Though most abundant in young  
5 neurons extending neurites, it is first expressed earlier in neuronal ontogeny, including in VZ cells (Gravel et al., 1988). The 4.5 kb 5' flanking region from the CNPase gene contains those sequence elements responsible for specifying CNPase expression to developing oligodendrocytes, and for regulating its expression as a function of growth (Scherer et al., 1994; Gravel et al., 1998). Transgenic mice with  
10 the 4.5 kb 5' flanking region fused to a nuclear *lacZ* reporter manifested expression of the CNPase promoter-driven transgene only within developing oligodendrocytes within the central nervous system, suggesting that CNPase promoter was expressed by oligodendrocytic precursor cells, as well as their young neuronal progeny.

The oligodendrocytic specificity and early expression of the CNPase  
15 promoter was capitalized on to use it as a marker for new oligodendrocytes and their parental precursors. This was done by coupling the CNPase promoter to the red-shifted mutant form of green fluorescent protein (Chalfie et al., 1994; Heim and Tsien, 1996), and transfecting both embryonic and adult forebrain subcortical cells with the resulting construct. These cells were successfully transfected with several  
20 liposomal techniques, including Lipofectin (Life Technologies, Grand Island, New York), and Effectin (Qiagen), as well as with an adenoviral vector bearing GFP. These techniques are particularly appropriate for transfecting CNP:GFP into large, mixed cell populations.

Two days after transfection, CNP2 expressing white matter cells,  
25 largely either young oligodendrocytes or their oligodendrocyte-specified progenitors, were then identified on the basis of their GFP fluorescence to blue excitation. This approach allowed for the identification and observation, alive and in real time, of VZ precursor cells and their neuronal daughters, derived from both fetal and adult brain. The brilliant fluorescence of these oligodendrocyte-driven GFP<sup>+</sup> precursor cells  
30 permitted their isolation and selective enrichment by fluorescence activated cell sorting (FACS), which yielded cell populations highly enriched in young

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oligodendrocytes and their precursors; of the latter, many proceeded to differentiate as neurons in the days following their preparation.

The cells separated by the method of the present invention may be used in both basic analyses of oligodendrocyte precursor growth control, as well as in directly applied studies of their transplantability and engraftment characteristics. This approach permits oligodendrocyte progenitor engraftment for the remyelination of demyelinated foci, in both ischemic and inflammatory demyelinating diseases, such as subcortical infarction, hypertensive leukoencephalopathy, and multiple sclerosis, respectively. The cells similarly can be used in support of the structural repair of the damaged central nervous system, such as in the traumatized brain, or the contoured, traumatized, or transected or spinal cord.

## EXAMPLES

### 15 Example 1 - Materials and Methods

#### *Plasmid construction*

*P/hCNP2:hGFP and P/hCNP2:lacZ* Humanized GFP (hGFP), a mutant form of GFP optimized for expression in human cells (Levy, et al., 1996, which is hereby incorporated by reference), was placed under the control of the human CNP2 promoter (Douglas, et al., 1992; Monoh, et al, 1993; and Gravel, et al., 1996). The human CNP gene had been isolated previously (Gravel, et al., 1996), by screening a human fibroblast genomic library with a cDNA probe for rat CNP1. The human CNP gene was then subcloned into pBluescript, and the resultant plasmid was designated SK/hgCNP. This plasmid was digested with BglII and XhoI to delete much of the gene downstream of the promoter region. The remaining BglII and XhoI ends were then filled-in and blunt-end ligated, yielding plasmid SK/P1P2hCNP, in which both BglII and XhoI were regenerated. A 1123 bp XhoI-XhoI fragment containing SV40 SD/SA-GFP<sub>h</sub>-SV40 poly(A) was then excised from pTα 1:hGFP (Wang, et al., 1998), and subcloned into XhoI-digested SK/P1P2hCNP to generate the plasmid P1P2hCNP:hGFP. The orientation of the hGFP insert was then determined by restriction enzyme mapping.

To construct *pP/hCNP2:hGFP*, the T $\alpha$ 1 tubulin promoter region was excised from *pP/T $\alpha$ 1:hGFP* using XbaI, and replaced with the hCNP2 promoter obtained by digesting SK/hgCNP with XbaI. The orientation of hCNP2 insert was also determined by restriction enzyme mapping. Similarly, *P2hCNP:lacZ* was constructed by removing the T $\alpha$ 1 promoter from the T $\alpha$ 1:*lacZ* (Wang, et al., 1998) with XbaI, and replacing it with the XbaI-XbaI fragment containing the hCNP2 promoter. *P/CMV:hGFP* was constructed as previously reported Wang, et al., 1998).

*Adult human brain white matter dissociation and culture.*

Adult human brain tissues obtained in the course of lobectomy of epilepsy patients was used here. The tissues were collected in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS. The white matter was dissected from the rest of the tissue, cut into pieces of roughly 2 mm on edge, or 8 mm<sup>3</sup>, and rinsed twice with PIPES solution (120 mM NaCl, 5 mM KCl, 25 mM glucose, 20 mM PIPES). It was then digested in pre-warmed papain/PIPES solution (11.4 U/ml papain, Worthington) and DNase I (10 U/ml, Sigma), on a rocking shaker for 1 hr at 37°C. The tissue was then collected by centrifuging at 200g in an IEC Centra-4B centrifuge, resuspended in DMEM/F12/N2 with DNase I (10 U/ml), and incubated for 15 min at 37°C. The samples were again spun and their pellets recovered in 2 ml of DMEM/F12/N2. They were then dissociated by sequentially triturating for 20, 10, and 5 times, respectively, through 3 glass Pasteur pipettes fire polished to decreasing bore diameters. Undissociated tissue pieces were eliminated by passage through a fine 40  $\mu$ m mesh. The cells were collected and rinsed once with DMEM/F12/N2 containing 20% platelet-depleted FBS (PD-FBS, Cocalico), to stop the enzymatic dissociation, and then resuspended at 1 X 10<sup>7</sup> cells/ml in DMEM/F12/N2 containing 10% FBS. The cell suspension was plated at 0.1 ml/dish into 35 mm Falcon Primaria plates coated with laminin (2  $\mu$ g/cm<sup>2</sup>) and incubated at 37°C in 5% CO<sub>2</sub>. After 4 hrs., an additional 0.7 ml of DMEM/F12/N2 with 2% PD-FBS was added into each plate. This medium was supplemented with IGF-1 (20 ng/ml; Sigma), FGF-2 (20 ng/ml; Sigma), NT-3 (20 ng/ml; Regeneron), and BrdU (10 nM). Following three days of incubation the cultures were switched to serum-free DMEM/F12/N2, with maintained growth factor and BrdU supplementation. Cultures were transfected at 7 DIV.

### *Transfection*

All plasmid constructs were introduced into the cultured cells by liposomal transfection, as described Wang, et al., 1998. Briefly, 6 to 7 days after plating, each 35 mm dish received mixture of 2 µg of plasmid DNA and 10 µl of lipofectin in OPTI-MEM (GIBCO). The cells were incubated at 37°C in 5% CO<sub>2</sub>/95% air for 6 hrs. The transfections were terminated with in DMEM/F12/N2 with 10% PD-FBS. After 2 hrs, the cells were returned into serum free DMEM/F12/N2 with PDGF AA and bFGF. Imaging for hGFP was first done 2 days after transfection, and daily thereafter, using an Olympus IX70 epifluorescence microscope. The greatest number and proportion of GFP<sup>+</sup> cells were observed 6-7 days after transfection; cultures were therefore sorted at that timepoint.

### *Flow cytometry and sorting*

Flow cytometry and sorting of hGFP<sup>+</sup> cells was performed on a FACS Vantage (Becton-Dickinson). Cells ( $5 \times 10^6$  /ml) were analyzed by light forward and right-angle (side) scatter, and for GFP fluorescence through a  $530 \pm 15$  nm bandpass filter, as they traversed the beam of an argon ion laser (488 nm, 100 mW). *P/hCNP2:lacZ*-transfected control cells were used to set the background fluorescence; a false positive rate of  $0.02 \pm 0.05\%$  was accepted so as to ensure an adequate yield. For the test samples transfected with *P/hCNP2:hGFP*, cells having fluorescence higher than background were sorted at 3000 cells/sec. Sorted cells were plated onto laminin-coated 24-well plates, into DMEM/F12/N2 containing PDGF AA and FGF2, each at 20 ng/ml, and BrdU. After 4 days, some plates were fixed for immunocytochemistry, and the remainder switched to DMEM/F12/N2 containing 10% PD-FBS. After an additional 3 weeks *in vitro*, the sorted cells were stained for either CNP, O4, TuJ1, or GFAP-immunoreactivities; each was double-stained for BrdU as well.

### *Data Analysis*

Experimental endpoints included the proportion of A2B5, O4, CNP, GFA, and TuJ1-immunoreactive cells in the total sorted population (all nominally

GFP<sup>+</sup> following sorting), as a function of time after FACS. At each sampled time-point, the respective proportions of A2B5<sup>+</sup>, O4<sup>+</sup>, CNP<sup>+</sup>, GFA<sup>+</sup>, and TuJ1<sup>+</sup> cells were compared to each other, and to unsorted controls that were similarly dispersed but replated without sorting (after adjusting their cell densities to those of the post-FACS sorted pool). For each combination of treatment (sorted or unsorted), time-point (4 days and 3-4 weeks after FACS), and immunolabel (A2B5, O4, CNP, TuJ1, and GFA), the number of stained and unstained cells were counted in 10 randomly chosen fields, in each of 3 triplicate cultures.

#### 10 *Immunocytochemistry*

Cells were immunostained live for A2B5 or O4, or following fixation with 4% paraformaldehyde, for CNP, TuJ1, GFAP, or BrdU. For A2B5 or O4 immunocytochemistry, plates were washed twice with DMEM/F12/N2, then blocked with DMEM/F12/N2 containing 5% normal goat sera (NGS) for 10 min at 4°C. Monoclonal antibody (MAb) A2B5 (clone 105, ATCC) was used as an undiluted culture supernatant, and mouse MAb O4 (Boehringer Mannheim) was used at 1:200. Both were applied in DMEM/F12/N2 for 30 min at 4°C. The plates were then washed with three changes of cold HBSS containing 1% NGS. The secondary antibody, Texas Red-conjugated goat anti-mouse IgM was used at a dilution of 1:50 for 30 min at 4°C. The cells were then washed and fixed with cold 4% paraformaldehyde for 10 min, washed, mounted in SlowFade and observed using an Olympus IX70 equipped for epifluorescence. Immunocytochemistry for GFAP and TuJ1 was performed according to described methods (Wang, et al., 1998), as was that for TuJ1 and BrdU (Luskin, et al., 1997).

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#### **Example 2 - Dissociates of Adult Human White Matter Harbored a Pool of Bipolar, A2B5<sup>+</sup> Cells**

To fully characterize the cell phenotypes resident in adult human white matter, papain dissociates of surgically-resected frontal and temporal capsular white matter were obtained from 8 patients. These included 4 males and 4 females, who ranged from 24-65 years old. Three patients had temporal lobe resections for medication-refractory epilepsy; 2 were subjected to parenchymal excision during

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meningioma resection, 2 samples were taken during aneurysmal repair, and 1 was taken from the non-neoplastic approach to a histologically benign ganglioglioma. The monolayer cultures resulting from these white matter dissociations were stained after 5-7 days *in vitro* (DIV) for either of 2 oligodendrocytic markers, that included the epitopes recognized by the A2B5 and O4 antibodies. Additional, matched cultures were stained after 14 DIV, for either A2B5 or O4, for oligodendrocytic CNP protein, or for either neuronal ( $\beta$ III-tubulin) or astrocytic (glial fibrillary acidic protein, GFAP) target antigens.

In the 14 DIV dissociates of subcortical white matter,  $48.2 \pm 10.7\%$  of the plated cells expressed the oligodendrocytic epitope recognized by MAb O4 ( $n=3$  patients, with a combined total of 935 O4<sup>+</sup> cells among 2041 scored white matter cells) (Figure 2). Similarly,  $49.9 \pm 4.9\%$  were immunoreactive for oligodendrocytic CNP protein. Another  $7.3 \pm 3.2\%$  expressed glial fibrillary acidic protein (GFAP). Double-labeling of selected plates revealed that the O4<sup>+</sup> and CNP<sup>+</sup> pools were largely overlapping, with a small proportion of CNP<sup>+</sup>/O4<sup>-</sup> cells. In contrast, the GFA<sup>+</sup> cells only rarely included O4<sup>+</sup> examples. A small proportion of TuJ1<sup>+</sup> neurons ( $5.2 \pm 2.2\%$ ) was also observed. Through 30 DIV, the proportions of oligodendrocytes and neurons in these cultures remained roughly stable, with  $51.3 \pm 7.0\%$  O4<sup>+</sup> cells and  $6.0 \pm 2.1\%$  TuJ1<sup>+</sup> cells, respectively. In contrast, the proportion of GFA-defined astrocytes in these cultures increased from  $7.3 \pm 3.2\%$  at 14 DIV to  $15.9 \pm 1.4\%$  at 30 DIV ( $p < 0.01$  by Student's t-test). At each time point, the uncharacterized remainder of the total cell population included endothelial cells and microglia, whose numbers we did not quantify further.

Notably, a distinct population of small bipolar cells, that expressed A2B5, but which otherwise expressed neither neuronal nor oligodendrocytic phenotypic markers, was observed; these constituted  $1.8 \pm 0.4\%$  ( $n=5$  patients) of all cultured white matter cells at 7 days. However, these cells became scarcer with time *in vitro*; by 30 DIV, A2B5<sup>+</sup> cells constituted  $<0.1\%$  of the total cultured cell pool.

**Example 3 - The CNP2 Promoter Targeted GFP Expression to a Bipolar, A2B5<sup>+</sup> Phenotype**

To identify either oligodendrocyte progenitor cells or their immature progeny, white matter dissociates were next transfected with plasmids encoding P/hCNP2:hGFP. Within 4 days after transfection with P/hCNP2:hGFP, a small proportion of GFP<sup>+</sup> cells were noted. These were invariably small, bipolar cells, and constituted <1% of the total cell pool (Figure 3). After an additional 4-7 days *in vitro*, the cultures were immunostained for one of 3 oligodendrocyte lineage markers, which included A2B5, O4, and CNP protein, or for either astrocytic GFAP or neuronal  $\beta$ III-tubulin. At that point, the cells could generally be described as A2B5<sup>+</sup>/O4<sup>+</sup>/GFAP<sup>-</sup>/TuJ1<sup>-</sup>: 62.5  $\pm$  8.8% of P/hCNP2:hGFP<sup>+</sup> cells expressed A2B5-IR, 21.1  $\pm$  7.5% were O4<sup>+</sup>, and another 7.3  $\pm$  3.2% expressed astrocytic GFAP. None were recognized by MAb TuJ1, which targets neuronal  $\beta$ III-tubulin (Menezes, et al., 1994, which is hereby incorporated by reference). Thus, within the first 7-10 days in culture, P/hCNP2:hGFP selectively identified a population of bipolar, A2B5<sup>+</sup> cells. When followed over the weeks thereafter, most of these P/hCNP2:hGFP<sup>+</sup> cells developed into oligodendrocytes, that could be recognized by their small, multipolar, heavily branching profiles. Indeed, by 4 weeks, most P/hCNP2:hGFP<sup>+</sup> cells expressed O4, while only rare cells (<1%) continued to express A2B5-IR.

**Example 4 - P/hCNP2:hGFP-identified Cells were Mitotic *in vitro***

Among white matter dissociates continuously exposed to BrdU and transfected with pP/hCNP2:hGFP on day 4 *in vitro*, 55  $\pm$  14.8% of the resultant P/hCNP2:hGFP<sup>+</sup> cells incorporated BrdU by day 7 (n=30 plates, derived from 3 patients) (Figure 3). Similarly, 43.1  $\pm$  9.1% (n=5 plates) of the A2B5<sup>+</sup> cells in matched plates incorporated BrdU over the same time period. Morphologically, essentially all of these A2B5<sup>+</sup> and BrdU<sup>+</sup> cells were bipolar at 1 week (Figure 2). In contrast, the large majority of morphologically mature oligo-dendrocytes failed to incorporate BrdU *in vitro*: Only 2.1  $\pm$  1.1% of O4<sup>+</sup> cells labeled with BrdU to which they were exposed during the first week in culture, and these few O4<sup>+</sup> cells may have just arisen from A2B5<sup>+</sup> forebears.

**Example 5 - FACS Segregated P/hCNP2:hGFP<sup>+</sup> Cells as Bipolar, A2B5<sup>+</sup> Progenitors**

Using sorting criteria intended for cell-type purification, the P/hCNP2-driven GFP<sup>+</sup> cells were then enriched and cultured separately (Figure 4). Immediately after FACS, P/hCNP2:hGFP-separated cells largely expressed A2B5-IR. Furthermore, the majority of these A2B5<sup>+</sup> cells were found to have incorporated BrdU from their culture medium prior to FACS, indicating their mitogenesis *in vitro* (Figure 5). Within the week after sorting, and with concurrent transfer to higher serum media, most of the sorted cells developed O4 expression, and lost A2B5-IR.

Notably, P/hCNP2:hGFP-separable cells were not rare. Among 7 patients whose white matter dissociates were transfected with P/hCNP2:hGFP,  $0.59 \pm 0.1\%$  of all subcortical cells expressed the transgene and could be separated on that basis. As a result, typically  $>2000$  pCNP2:hGFP<sup>+</sup> cells ( $2382 \pm 944$ ) were obtained from sorts that averaged 352,000 gated cells (Figure 6). In this regard, net transfection efficiency, determined using P/CMV:hGFP, was  $13.5 \pm 2.2\%$  ( $n=3$ ), suggesting that roughly 1 cell in 8 was successfully transfected with the promoter-driven reporter. This in turn suggested that oligodendrocyte progenitor cells comprise at least 4% ( $0.59\% \times 1/0.135=4.37\%$ ) of all cells in the subcortical white matter.

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**Example 6 - P/hCNP2:hGFP<sup>+</sup>-sorted Cells Matured Largely, but not Exclusively, into Oligodendrocytes**

The majority of CNP2-sorted cells developed and matured as oligodendrocytes. By 3 weeks after FACS,  $74.1 \pm 7.7\%$  of these cells expressed oligodendrocytic CNP protein; a matched sample of sorted cells stained after 3 weeks *in vitro* for O4 yielded  $66.3 \pm 6.8\%$  O4-IR cells, most of which co-labeled for the more mature marker galactocerebroside (Figure 7). Nonetheless, concurrent development of non-oligodendrocytic phenotypes was also noted after FACS purification, albeit at lower frequency than oligodendrocytes: Immediately after sorting,  $6.5 \pm 5.4\%$  of the sorted cells expressed GFAP, and  $11.0 \pm 4.6\%$  were GFAP<sup>+</sup> by 3 weeks *in vitro*. These were not simply false-positive contaminants, in that most were observed to express P/hCNP2:hGFP fluorescence. No P/hCNP2:hGFP<sup>+</sup> neurons, as defined by concurrent TuJ1/ $\beta$ III-tubulin IR, were observed immediately prior to

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FACS, but  $7.5 \pm 4.4\%$  of P/hCNP2:hGFP-sorted cells did mature into TuJ1<sup>+</sup> neurons in the week thereafter. These TuJ1<sup>+</sup> cells were confirmed visually as expressing P/hCNP2:hGFP. The presence of these sporadic P/hCNP2:hGFP<sup>+</sup> neurons and astrocytes after FACS suggests that P/hCNP2-defined progenitors may retain some degree of multilineage potential in vitro.

These data indicate that the adult human subcortex harbors a population of residual, mitotically-competent oligodendrocyte progenitor cells. The cells constitute a discrete population of bipolar blasts, distinct from mature oligodendrocytes. The progenitors were mitotically competent, and as such, distinct from the much larger population of mature, apparently post-mitotic oligodendrocytes. These cells were antigenically immature (A2B5<sup>+</sup>/O4<sup>-</sup>) when isolated, but matured (O4<sup>+</sup>/O1<sup>+</sup>) over several weeks in culture. Cell-specific targeted reporting, achieved by transfecting the overall white matter pool with plasmids of GFP placed under the control of the early promoter for oligodendrocytic CNP, allowed the live-cell identification of these progenitor cells. This in turn provided a means for their isolation and purification, by fluorescence-activated cell sorting based on P/hCNP2-driven GFP expression.

*The nature of the adult white matter progenitor pool*

Intriguingly, these CNP2-defined oligodendrocytic progenitors were not rare: By the present sorting criteria, they constituted as many as 4% of total white matter cells. The abundance and ubiquity of these cells suggest that they may play an important role in the maintenance and function of the normal adult white matter. In rats, a substantial proportion of the white matter cells population is cycling at any one time. Although this issue has not been well-studied in humans, the presence of such a large progenitor pool in humans suggests that some degree of oligodendrocytic turnover may be occurring in the human subcortical white matter, with the replacement of damaged or dysfunctional postmitotic oligodendrocytes by progenitor-derived replacements. Such speculation notwithstanding, the normative roles of the white matter progenitor pool in both healthy and pathologic brain function remains unclear, and begs further study.

*Ontogeny and lineage of parenchymal oligodendrocytic precursors*

Neural precursor cells are widespread in the subependymal zone (SZ) of the forebrain ventricular lining (Goldman, et al., 1983; Goldman, et al., 1992; Kirschenbaum, et al., 1995; Lois, et al., 1993; Luskin, M. B., 1993; and Morshead, et al., 1994). Whether the P/hCNP2:hGFP-defined subcortical precursors described here are co-derived with the SVZ-based progenitor pool is unknown. It is also unclear whether the P/hCNP2-defined precursors constitute committed oligodendrocyte precursors, or whether they are more intrinsically pluripotent, and generate given lineages as a function of the environment to which they are exposed. The latter possibility is suggested by the small proportion of P/hCNP2:hGFP<sup>+</sup> cells that were found to be GFAP<sup>+</sup> astrocytes upon immunostaining; many of these never developed expression of any oligodendrocytic marker, and appeared instead to be astrocytes. This suggests that the P/hCNP2-defined progenitor pool may constitute a bipotential astrocyte-oligodendrocyte progenitor, which may yield largely oligodendrocytic progeny by virtue of the culture conditions employed. As such, this cell type may well be analogous to its A2B5-defined counterpart in the developing optic nerve (Butt, et al., 1993; Colello, et al., 1995; and Noble, et al., 1992). Whether these cells might also be competent to generate neurons remains unclear. No P/hCNP2:hGFP<sup>+</sup> cells were found to express neuronal TuJ1 in unsorted white matter cultures, out of >2000 hGFP<sup>+</sup> cells studied. Nonetheless, a small number of TuJ1<sup>+</sup> cells were noted to develop in P/hCNP2:hGFP-sorted cultures, and these TuJ1-defined neurons were confirmed as P/hCNP2:hGFP<sup>+</sup>, rather than nonfluorescent contaminants of the sorts. Thus, with time *in vitro*, particularly in the mitogenic FGF2/PDGF/NT3 environment provided here, it remains possible that these cells retain or regain a capacity for multilineage differentiation, as in development (Davis, et al., 1994 and Williams, et al., 1991). Importantly, only P/hCNP2:hGFP-defined cells were noted to mature as neurons after high-grade enrichment by sorting. Thus, the multilineage potential of these cells might be preferentially exercised after their isolation from other cell types, in low density culture. As such, the relative fidelity to oligodendrocytic phenotype by P/hCNP2:hGFP-defined cells in the initial white matter dissociates, prior to FACS, might reflect an initial restriction of progenitor phenotype by paracrine and/or density-dependent influences *in vitro*.

*Implantation for the treatment of demyelinating diseases*

The high-yield acquisition of oligodendrocyte progenitor cells from the adult human brain may allow growth and differentiation requirements specific to these cells to be better defined. Such information may enable their endogenous activation and/or engraftment. The potential use of these cells as substrates for induced remyelination suggests therapeutic strategies appropriate to a variety of white matter diseases. These potential therapeutic targets include ischemic demyelination, as in subcortical lacunar infarction and hypertensive leukoencephalopathy, post-inflammatory demyelinations, such as radiation necrosis and remitted multiple sclerosis, as well as the degenerative and metabolic leukodystrophies.

Taken together, these observations suggest that oligodendrocyte progenitors persist in relative abundance in the adult human white matter. P/hCNP2:hGFP-based FACS permits their viable harvest, in sufficient numbers and purity to enable their potential use in cell-based therapeutic strategies.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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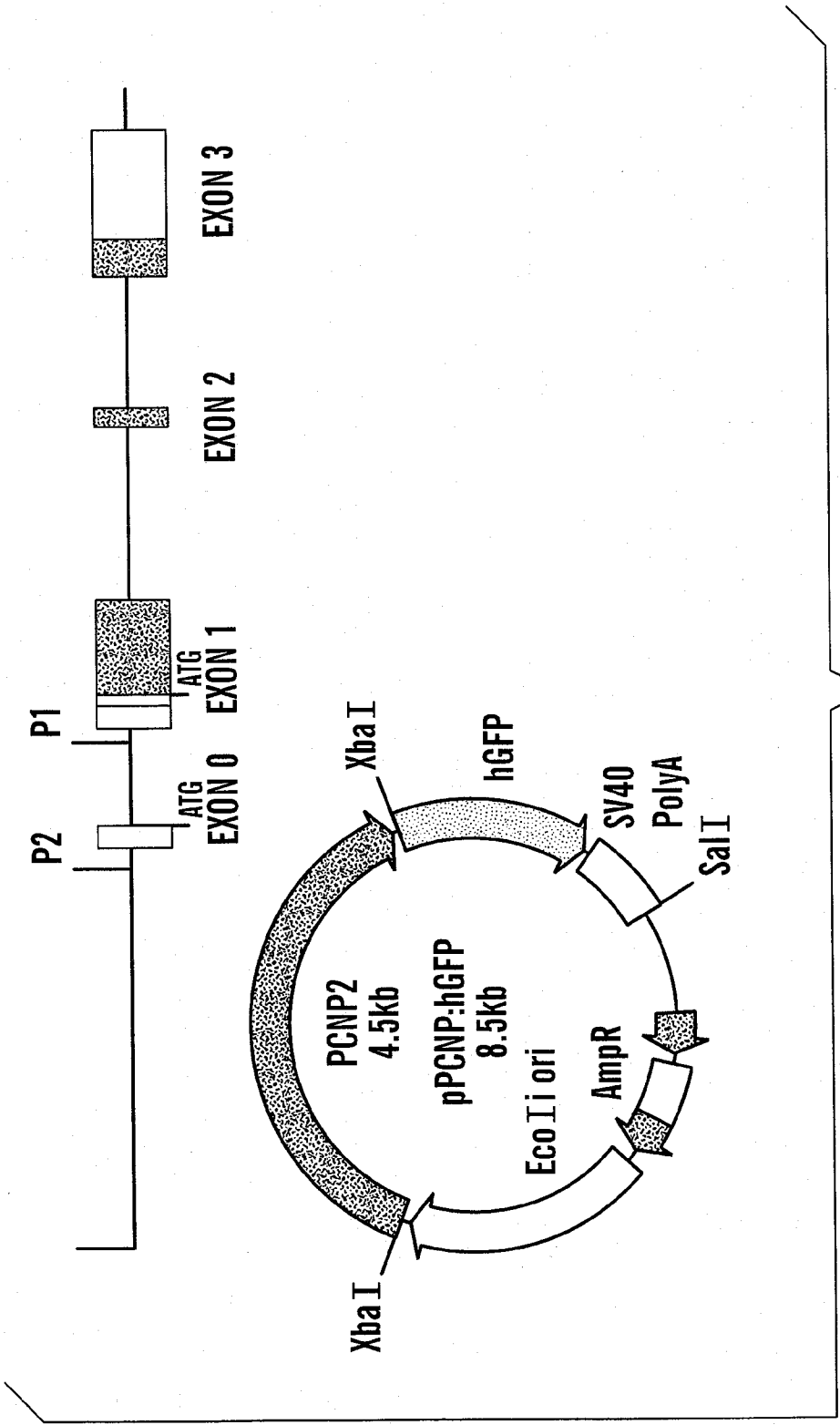
**WHAT IS CLAIMED IS:**

1. A method of separating oligodendrocytes or progenitor cells thereof from a mixed population of cells comprising:
  - 5 selecting a promoter which functions only in said oligodendrocytes or progenitor cells thereof;
  - introducing a nucleic acid molecule encoding a fluorescent protein under control of said promoter into the mixed population of cells;
  - allowing the oligodendrocytes or progenitor cells thereof to
  - 10 express the fluorescent protein; and
  - separating the fluorescent cells from the mixed population of cells, wherein said separated cells are said oligodendrocytes or progenitor cells thereof.
- 15 2. The method of claim 1, wherein said introducing comprises viral mediated transduction of said oligodendrocytes and progenitors thereof.
3. The method of claim 2, wherein said viral mediated transduct comprises adenovirus-mediated transduct.
- 20 4. The method of claim 1, wherein said introducing comprises electroporation.
5. The method of claim 1, wherein said introducing comprises
- 25 biolistic transformation.
6. The method of claim 1, wherein said introducing comprises liposomal mediated transformation of said plurality of cells.
- 30 7. The method of claim 1, wherein said separating comprises fluorescence activated cell sorting.
8. The method of claim 1, wherein said promoter is a CNP-P1 promoter, a CNP-P2 promoter, a CNP-P1+P2 promoter, a NCAM promoter, a myelin

basic protein promoter, a JC virus minimal core promoter, a myelin-associated glycoprotein promoter, or a proteolipid protein promoter.

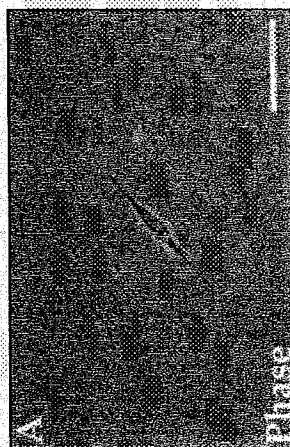
- 5        9.        The method of claim 8, wherein the promoter is a P/CNP2 promoter.
- 10       10.       The method according to claim 1, further comprising:  
             identifying the cells of said mixed population of cells that are  
             fluorescent, wherein the identifying step is after the allowing step.
- 15       11.       The method of claim 1, wherein the mixed population of cells  
             is in a tissue.
12.       The method of claim 11, wherein the tissue is brain tissue.
13.       The method of claim 11, wherein the tissue is spinal cord  
             tissue.
- 20       14.       The method of claim 11, wherein the mixed population of cells  
             is in a cell culture.
15.       The method of claim 1, further comprising:  
             transplanting the separated cells into a subject.
- 25       16.       The method of claim 1, wherein the oligodendrocytes or  
             progenitor cells thereof are human in origin.
17.       An enriched or purified preparation of isolated postnatal  
             oligodendrocytes.
- 30       18.       The enriched or purified preparation of isolated  
             oligodendrocytes of claim 17 which are human.

19. An enriched or purified preparation of isolated oligodendrocyte progenitor cells.
- 5           20. The enriched or purified preparation of isolated oligodendrocyte progenitor cells of claim 19 which are human.



**FIG. 1**

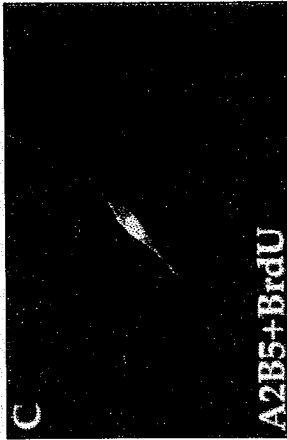
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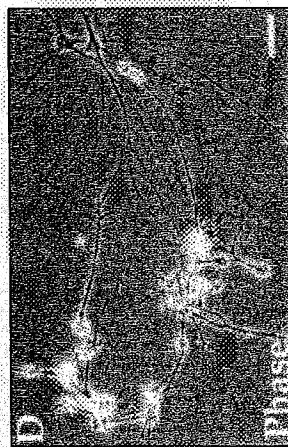
**FIG. 2A**



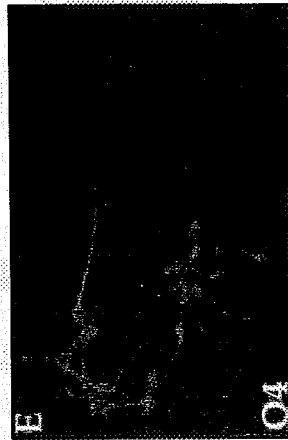
**FIG. 2B**



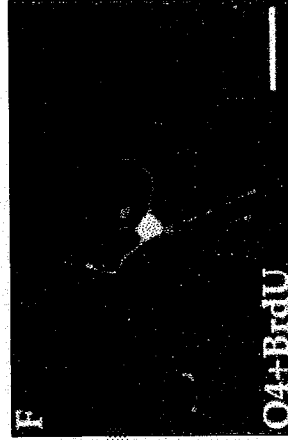
**FIG. 2C**



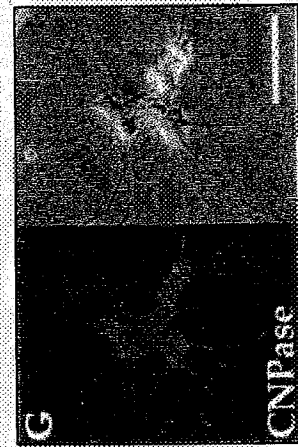
**FIG. 2D**



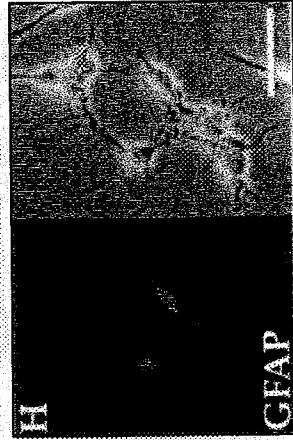
**FIG. 2E**



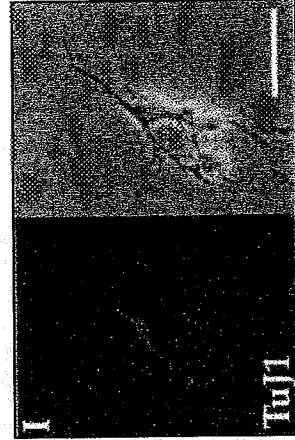
**FIG. 2F**



**FIG. 2G**

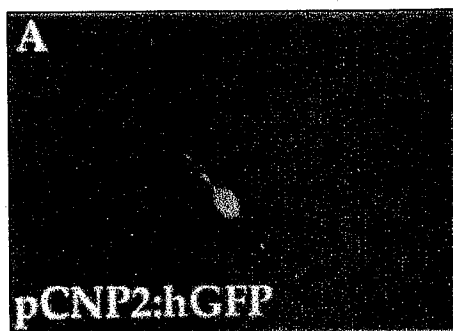
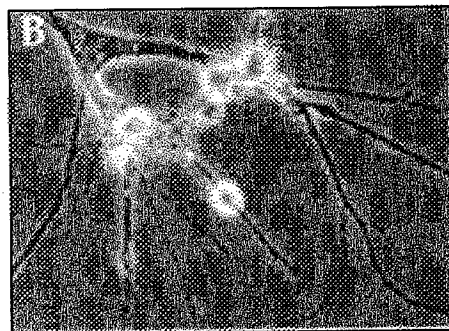
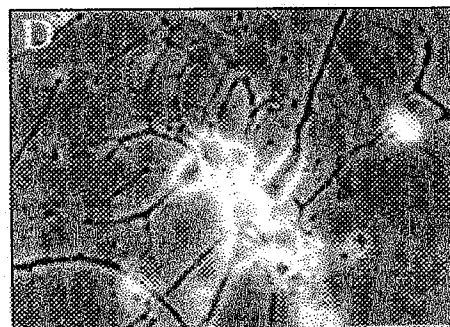
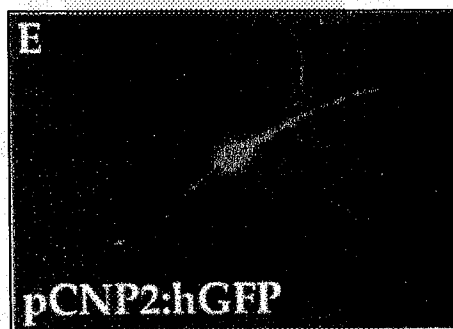
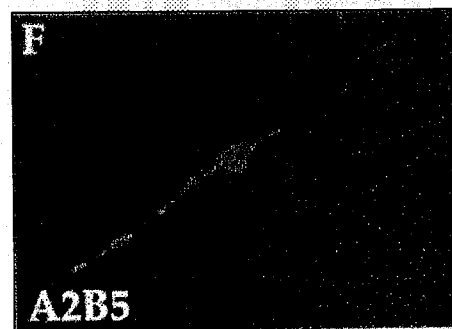
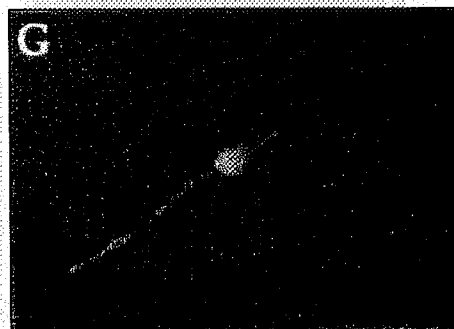
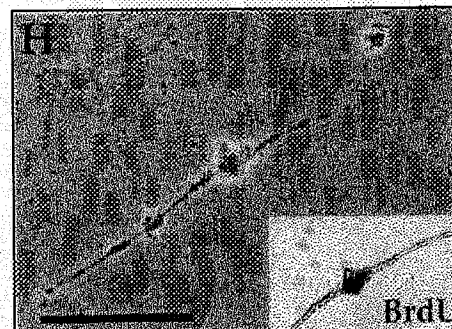


**FIG. 2H**



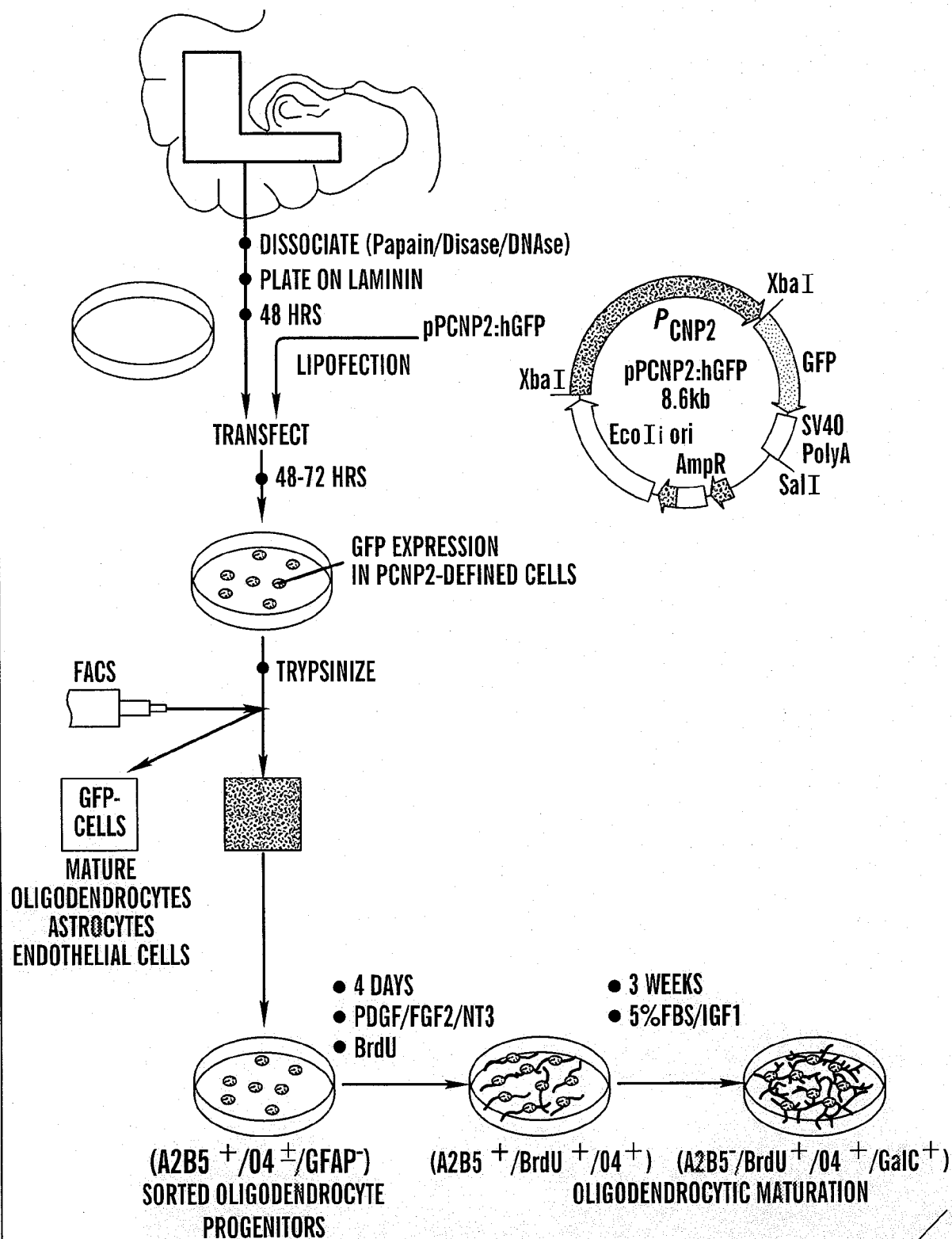
**FIG. 2I**

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**FIG. 3A****FIG. 3B****FIG. 3C****FIG. 3D****FIG. 3E****FIG. 3F****FIG. 3G****FIG. 3H**

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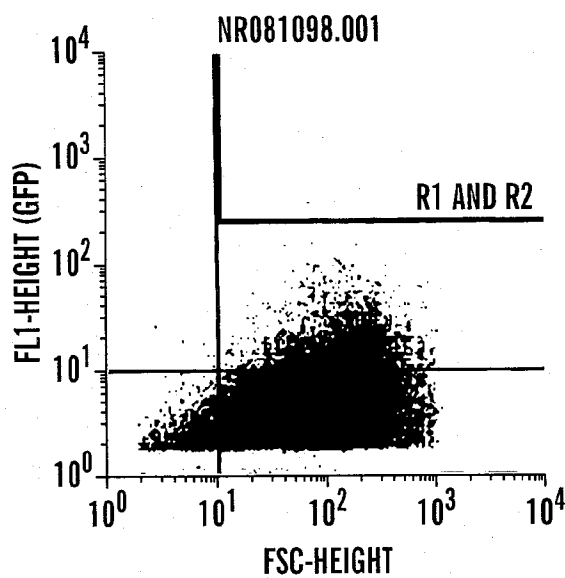
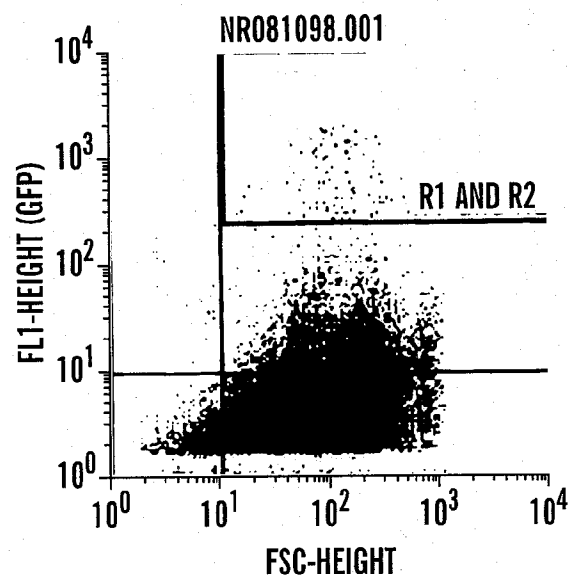
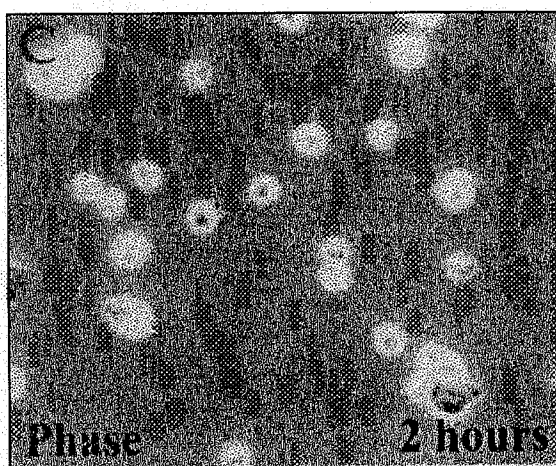
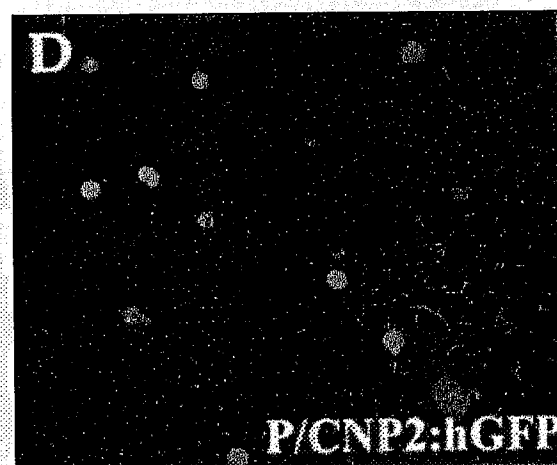
# IDENTIFICATION AND ENRICHMENT OF OLIGODENDROCYTE PROGENITOR CELLS FROM ADULT HUMAN FOREBRAIN

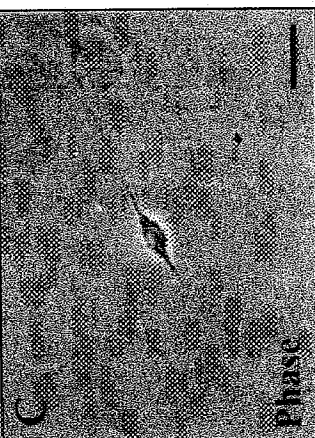


**FIG. 4**

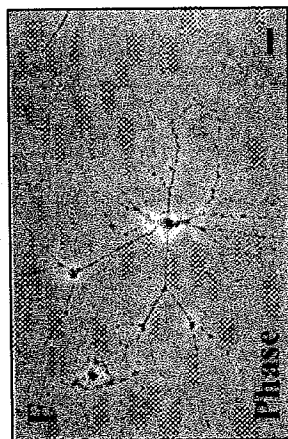
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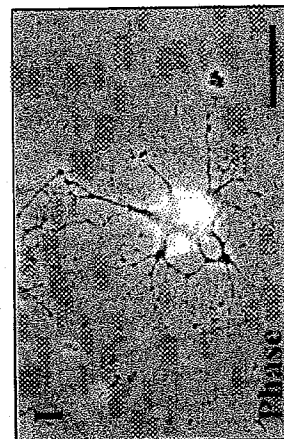
**FIG. 5A****FIG. 5A****FIG. 5C****FIG. 5D**



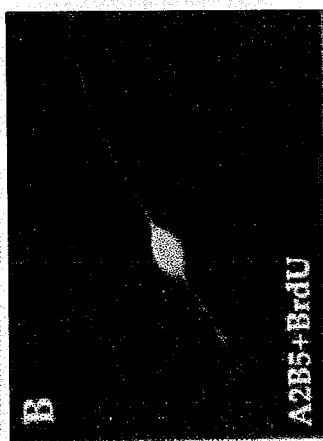
**FIG. 6C**



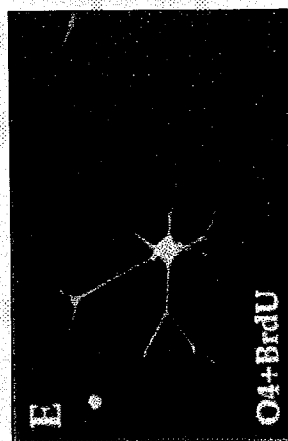
**FIG. 6F**



**FIG. 6I**



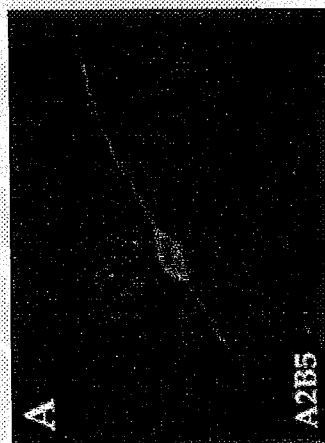
**FIG. 6B**



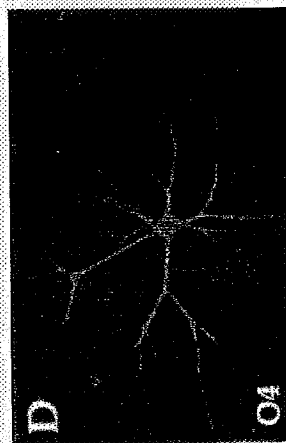
**FIG. 6E**



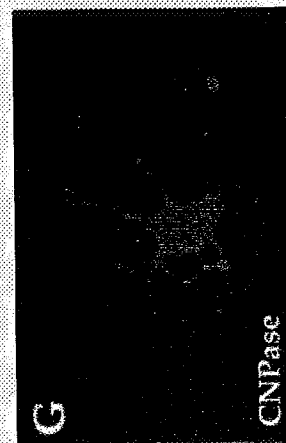
**FIG. 6H**



**FIG. 6A**

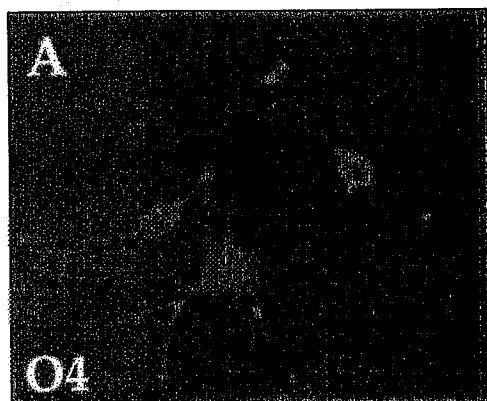


**FIG. 6D**

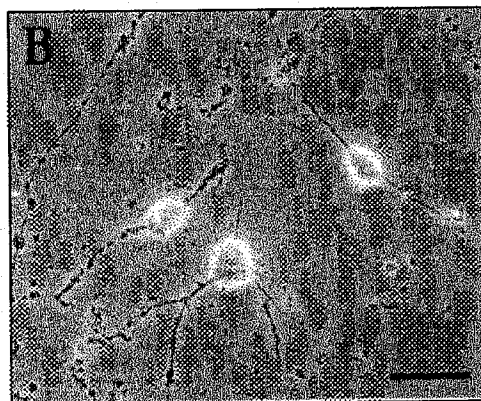


**FIG. 6G**

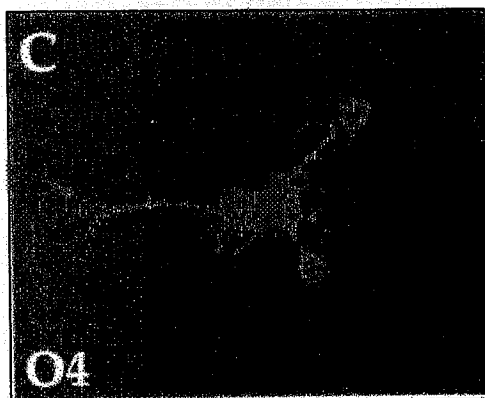
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**FIG. 7A**



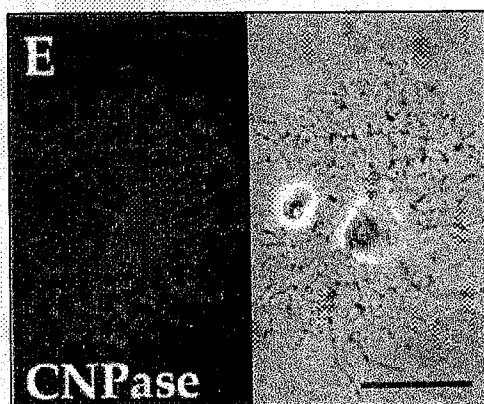
**FIG. 7B**



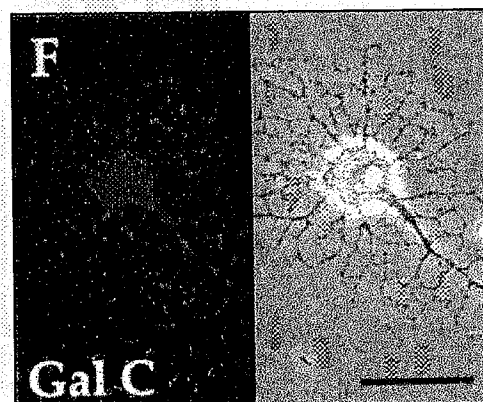
**FIG. 7C**



**FIG. 7D**



**FIG. 7E**



**FIG. 7F**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 5/10, A61K 35/30, A61P 25/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/23571</b> <b>(43) International Publication Date:</b> 27 April 2000 (27.04.00)
<b>(21) International Application Number:</b> PCT/US99/24326 <b>(22) International Filing Date:</b> 19 October 1999 (19.10.99)  <b>(30) Priority Data:</b> 60/104,809           19 October 1998 (19.10.98)   US 09/282,239           31 March 1999 (31.03.99)       US  <b>(71) Applicant:</b> CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).  <b>(72) Inventors:</b> GOLDMAN, Steven, A.; 7 Hillcrest Court, South Salem, NY 10590 (US). WANG, Su; Apartment 31, 441 Lawn Avenue, Palisades Park, NJ 07650 (US).  <b>(74) Agents:</b> GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		<b>(81) Designated States:</b> CA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 24 August 2000 (24.08.00)
<b>(54) Title:</b> A METHOD FOR ISOLATING AND PURIFYING OLIGODENDROCYTES AND OLIGODENDROCYTE PROGENITOR CELLS  <b>(57) Abstract</b>  <p>The present invention is directed to a method of separating oligodendrocyte cells or progenitor cells thereof from a mixed population of cells. It comprises selecting a promoter which functions only in the oligodendrocyte cells or progenitor cells thereof, introducing a nucleic acid molecule encoding a fluorescent protein under control of that promoter into the mixed population cells, allowing the oligodendrocyte cells or progenitor cells thereof to express the fluorescent protein and separating the fluorescent cells from the mixed population cells, where the separated cells are the oligodendrocyte cells or progenitor cells thereof. The invention also relates to the isolated and enriched human oligodendrocyte cells or progenitor cells thereof.</p>		

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/24326

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/10 A61K35/30 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 32879 A (CORNELL RES FOUNDATION INC) 30 July 1998 (1998-07-30) page 10, line 34 -page 11, line 9 page 14, line 15 - line 21; claims the whole document ---	1-20
X	WO 96 38576 A (SIGNAL PHARM INC) 5 December 1996 (1996-12-05) claims ---	15, 17-20
X	WO 94 09119 A (NEUROSPHERES LTD) 28 April 1994 (1994-04-28) claims ---	15, 17-20
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

10 May 2000

Date of mailing of the international search report

23/05/2000

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Teyssier, B

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/24326

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SCHERER S S ET AL.: "Differential regulation of the 2',3'-cyclic nucleotide 3'-phosphodiesterase gene during oligodendrocyte development" NEURON, vol. 12, no. 6, June 1994 (1994-06), pages 1363-1375, XP000906920 cited in the application the whole document</p> <p style="text-align: center;">---</p>	1-20
T	<p>ROY NEETA SINGH ET AL: "Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter." JOURNAL OF NEUROSCIENCE, vol. 19, no. 22, 15 November 1999 (1999-11-15), pages 9986-9995, XP000906926 the whole document</p> <p style="text-align: center;">-----</p>	1-20

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/24326

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 15 is directed to a mtehod of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the preparation.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/24326

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9832879 A	30-07-1998	AU 6245498 A	18-08-1998
WO 9638576 A	05-12-1996	US 5830651 A	03-11-1998
		AU 5959096 A	18-12-1996
WO 9409119 A	28-04-1994	AU 683023 B	30-10-1997
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		JP 8502172 T	12-03-1996
		NO 951378 A	07-04-1995
		WO 9416718 A	04-08-1994
		US 5750376 A	12-05-1998
		US 5980885 A	09-11-1999
		US 5851832 A	22-12-1998